Remarks

Upon entry of the foregoing amendment, claims 47-48 are pending in this application. Claims 1-46 have been canceled without prejudice or disclaimer. Applicants reserve the right to pursue claims directed to the canceled subject matter in a continuing or divisional application. Claims 47-48 are newly added and now under examination. Claim 47 is an independent claim and claim 48 depends from claim 47.

Support for new claims 47-48 is found, for example, on page 21, lines 12-16; page 23, line 4-7; page 29, line 26 through page 30, line 4; page 30, lines 8-10; page 40, lines 16-21 and lines 25-28; in particular, page 61, lines 13-17; and, elsewhere throughout the specification.

Regarding claims 47-48, the publication by Broun *et al.* (Plant Journal 13(2): 201-218 (1998)) is submitted herewith as evidence that, by using the methods disclosed in the specification, seed oils from *Brassica* may be obtained which have approximately 8% to 15% ricinoleic acid and about 59% oleic acid. The Broun publication is post-filing date work of the inventors and demonstrates that oil produced from transgenic *Brassica* seeds contains increased levels of ricinoleic fatty acid using methods disclosed in the instant application. See, Broun, page 204, Table 4, showing ricinoleic acid production in seeds using constructs TC12 (napin promoter/CFAH12) and TD29 (napin promoter/LFAH12). The Office should note that, according to the Broun publication, ricinoleic fatty acid is not found in seed oil from wild type *Brassica napus* seeds.

The instant specification discloses the methods may be practiced using the napin promoter (for example, page 39, lines 8-13) in *Brassica* spp (for example, page 40, lines 16-21) as is demonstrated in Broun. The Broun publication used the LFAH12 gene and the CFAH12 gene as do the methods of the specification (for example, CFAH12, Example 1, page 49, last paragraph; for example, LFAH12 gene, page 72, beginning line 11). Both Broun and the instant specification used *Agrobacterium*-mediated

transformation to obtain transformed plants (*Arabidopsis* and *Brassica* in Broun, page 204, left column, last paragraph; *Arabidopsis*, for example, specification page 49, lines 22-28).

Using the methods described in the specification in plant species contemplated in the specification, Applicants have demonstrated increased levels of ricinoleic fatty acid in seed oils from *Brassica*. Use of such methods to increase the fatty acid content of seed oils is disclosed at, for example, page 84, lines 11-19, and of ricinoleic acid content in particular, for example, at page 40, lines 25-28, of the specification. Thus, the specification clearly provides support for the invention as claimed.

The Office Action mailed April 20, 2004 (Paper No. Not Assigned) has been carefully reviewed. The amendments above and following remarks and arguments are made in response thereto. Reconsideration of this application and the timely allowance of the pending claims is respectfully requested. It is believed the amendments do not introduce new matter into the specification and entry is respectfully requested.

Status of the Claims and Response to Restriction Requirement

The finality of the restriction requirement and Office arguments pertaining thereto is acknowledged.

Objection to the Specification

Applicants acknowledge with thanks the withdrawal of the objection to the disclosure.

Objection to the Claims

At page 3 of the Office Action, the Office objects to claims 45 and 46 because they allegedly recite non-elected plant species. Claims 45 and 46 have been cancelled. New claims 47-48 have been amended to recite a plant genus/species and therefore new claims 47-48 are believed to be free of the objection. Reconsideration and withdrawal of the objection is respectfully requested.

The Rejection of Claims under 35 U.S.C. § 112, First Paragraph

At page 4 of the Office Action, the Office rejected claims 42-46 under 35 U.S.C. § 112, first paragraph because the claims allegedly lack written description support. The rejection is respectfully traversed.

Claims 42-46 have been canceled and new claims 47-48 added. Support for new claims 47-48 is described, above. The rejection is believed to be moot in view of new claims 47-48 and withdrawal of the rejection is respectfully requested.

Rejection under 35 U.S.C. § 112, first paragraph

At page 6 of the Office Action, the Office rejects claims 37-41 under 35 U.S.C. § 112, first paragraph because while being enabled for oil obtained from *Arabidopsis* that has been transformed with a nucleic acid sequence encoding a fatty acid hydroxylase, which comprises ricinoleic, lesquerolic, densipolic, and auricolic acid, allegedly does not provide enablement for oil from a plant that has been transformed with a nucleic acid sequence that encodes a fatty acid hydroxylase, wherein said oil has a hydroxylated fatty acid content that is increased compared to the hydroxylated fatty acid content from an oil obtained from seeds of the same plant that has not been transformed with a nucleic acid sequence that encodes a fatty acid hydroxylase. The rejection is respectfully traversed.

Claims 37-41 have been canceled and the rejection is believed to be moot since new claims 47-48 do not include a nucleic acid sequence encoding a hydroxylase. Withdrawal of the rejection is respectfully requested.

Rejection of claims 42-46 under 35 USC § 102(b) as being anticipated by, or alternatively under 35 USC § 103(a) as obvious over Badami *et al.*

At page 9 of the Office Action, the Office rejected claims 42-46 under 35 USC § 102(b) as anticipated by Badami *et al.* or, alternatively, under 35 USC § 103(a) as obvious over Badami *et al.* (Prog. Lipid Res. 19: 119 and 132-136 (1981) [Badami]). Claims 42-46

have been canceled and claims 47-48 newly added. The rejection is believed to be moot with respect to new claims 47-48. To the extent the rejection may remain pertinent, it is respectfully traversed.

The Office asserts the Badami document teaches seed oils from several plant families that have hydroxy fatty acids, including from the cruciferous species, *Lesquerella*, including ricinoleic , densipolic, lesquerolic acid and auricolic fatty acids and that Badami teaches a product that appears to be the same as, or an obvious variant of, the product set forth in the product by process claim. However, new claims 47-48 are directed to a seed oil obtained from the seeds of a *Brassica* spp.and the rejection is believed to be moot in view of the different subject matter of new claims 47-48.

It is noted that Badami discusses the hydroxyfatty acids occuring in *Cruciferae*. However, Badami does not teach or disclose the presence of ricinoleic acid in *Brassica* spp. Therefore, since Badami is silent on the presence of ricinoleic acid in *Brassica*, Badami cannot anticipate, or render obvious, the seed oils as now claimed. Reconsideration and withdrawal of the rejection is respectfully requested.

CONCLUSION

In view of the foregoing remarks, Applicants respectfully request withdrawal of all outstanding rejections and early notice of allowance to that effect. Should the Examiner believe that a telephonic interview would expedite prosecution and allowance of this application, she is encouraged to contact the undersigned at her convenience.

Except for issue fees payable under 37 C.F.R. § 1.18, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 C.F.R. §§ 1.16 and 1.17 which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account No.50-0310.

This paragraph is intended to be a **CONSTRUCTIVE PETITION FOR EXTENSION OF TIME** in accordance with 37 C.F.R. § 1.136(a)(3).

Attachments: Broun *et al.*, The Plant Journal 13(2): 201-210 (1998)

Respectfully submitted,

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A bifunctional oleate 12-hydroxylase: desaturase from *Lesquerella fendleri*

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Summary

***LFAH12*, an oleate 12-hydroxylase gene from *Lesquerella fendleri* (L.) was isolated on the basis of nucleotide sequence similarity to an oleate hydroxylase gene from *Ricinus communis* (L.). Transgenic *Arabidopsis* plants containing the *Lesquerella* gene under transcriptional control of the cauliflower mosaic virus 35S promoter accumulated ricinoleic, lesquerolic and densipolic acids in seeds, but not in leaves or roots. However, hydroxylase activity was detectable in crude extracts of vegetative tissues. The discrepancy between the presence of activity and the lack of hydroxy fatty acids suggests selective removal and breakdown of hydroxy fatty acids in vegetative organs. High levels of *LFAH12* mRNA accumulation did not lead to correspondingly high levels of protein accumulation, suggesting that accumulation of the hydroxylase may be controlled post-transcriptionally. Expression of the *L. fendleri* gene in transgenic plants of a *fad2* mutant of *Arabidopsis*, which is deficient in cytoplasmic oleate Δ12 desaturase activity, resulted in partial suppression of the mutant phenotype in roots. Thus, unlike the hydroxylase from *R. communis*, the *L. fendleri* enzyme has both hydroxylase and desaturase activities. Fusion of the 5′ flanking region of the *LFAH12* gene to the β-glucuronidase coding sequence resulted in a high level of early seed-specific expression of β-glucuronidase activity in transgenic *Arabidopsis* plants.**

Introduction

At least 14 plant species representing 10 families have been found to accumulate significant quantities of hydroxy fatty acids in seed storage lipids (van de Loo *et al.*, 1993). The synthesis of hydroxy fatty acids has been most intensively studied in seeds of castor (*Ricinus communis*) where ricinoleic acid (D-12-hydroxyoctadec-*cis*-9-enoic acid) accumulates to more than 80% of the fatty acids. Ricinoleate is synthesized from lipid-linked oleate by a membrane-bound hydroxylase (Bafor *et al.*, 1991; Moreau and Stumpf, 1981). Recently, the *CFAH12* gene encoding the castor oleate 12-hydroxylase has been cloned (van de Loo *et al.*, 1995). The gene product exhibited a high degree of amino acid sequence similarity to microsomal oleate 12 desaturases such as the *Arabidopsis* FAD2 desaturase. The high degree of sequence similarity suggests that in castor, and possibly other species that accumulate ricinoleic acid, the hydroxylase genes have evolved from desaturases. Expression of the castor gene in *Arabidopsis* seeds resulted in the accumulation of ricinoleate, densipoleate (D-12-hydroxyoctadec-*cis*-9,15-dienoic acid), lesqueroleate (D-14-hydroxyeicos-*cis*-11-enoic acid) and auricoleate (D-14-hydroxyeicos-*cis*-11,17-dienoic acid) (Broun and Somerville, 1997). It was inferred that densipoleate probably arose by desaturation of ricinoleate by a Δ15 desaturase (i.e. FAD3), and lesqueroleate and auricoleate may have arisen by elongation of ricinoleate and densipoleate, respectively, or by hydroxylation and desaturation of eicosenoate.

A common feature of all membrane-bound plant fatty acyl desaturases and the castor hydroxylase is the presence in their sequences of three clusters of histidine residues which are thought to act as ligands to the catalytic iron atoms which have been proposed to form a di-iron oxo group. Recent studies of the mechanism of action of di-iron desaturases and hydroxylases have suggested that the catalytic mechanism may be similar (Shanklin *et al.*, 1994, 1997). However, nothing is known about the structural differences which distinguish the two classes of enzymes. Extensive sequence information is now available for oleate 12-desaturases from various species (e.g. Lightner and Okuley, 1994; Okuley *et al.*, 1994). However, the sequence of the castor hydroxylase is the only sequence available for a diiron fatty acyl hydroxylase.

Species of the genus *Lesquerella* (Brassicacae) also accumulate hydroxy fatty acids. Their seed storage lipids typically contain ricinoleic acid, densipolic, lesquerolic and auricolic acids (Hayes *et al.*, 1995). Thus, we considered it likely that *L. fendleri* contained an enzyme closely related to the oleate hydroxylase from castor. We report here the isolation and characterization of *LFAH12*, the gene encoding the oleate 12-hydroxylase from *L. fendleri*. Analysis of the fatty acid composition of transgenic plants expressing the *L. fendleri* gene indicates that the enzyme exhibits both desaturase and hydroxylase activity. In addition, we present results showing that the promoter region of the *L. fendleri* hydroxylase gene can be used to cause high level expression of a foreign gene in transgenic plants.

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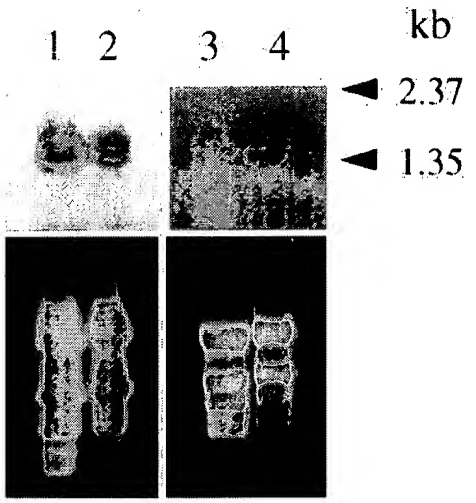

Figure 1. Northern blot of *L. fendleri* RNA probed with partial cDNAs encoding a putative oleate desaturase (*DLS2*, lanes 1,2) and a putative hydroxylase (*DLS1*, lanes 3,4).
Northern blots of total leaf RNA (lanes 1,3) and seed RNA (lanes 2,4) are shown at the top. A photograph of the ethidium bromide stained gels used to produce the filters is shown at the bottom.

The promoter appears to be seed specific and the timing of expression coincides with lipid accumulation in *Arabidopsis*.

Results

Cloning of the L. fendleri *oleate 12-hydroxylase*

Degenerate oligonucleotide primers were designed to encode short regions of the castor oleate 12-hydroxylase (CFAH12) and the *Arabidopsis* oleate 12-desaturase (FAD2). These primers (Deg1 and Deg2) spanned conserved histidine-rich motifs, separated by about 183 amino acid residues, which are the putative iron binding sites in CFAH12 and FAD2 (Shanklin *et al.*, 1994). First-strand cDNAs were obtained by reverse transcription of RNA from developing *L. fendleri* seeds, then used as templates in PCR reactions with primers Deg1 and Deg2. PCR products of the expected size were cloned and sequenced. Clones for two types of desaturase-like sequences, *DLS1* and *DLS2*, were identified and used as probes on Northern blots of *L. fendleri* leaf and seed RNA (Figure 1).

Both probes detected an RNA of about 1.4 kb. *DLS1* hybridized to an abundant transcript in RNA from developing seeds, but did not detect an RNA in leaf extracts. In contrast, *DLS2* detected transcripts in RNA from both seeds and leaves. Since hydroxylated fatty acids occur prominently in seeds but not leaves, these observations suggested that *DLS1* encoded part of an oleate 12-hydroxylase, whereas *DLS2* encoded part of an oleate 12-desaturase.

Table 1. Transgenic plant lines used in this study

Name	Parental line	Promoter	Insert	Source
SNTα133	WT	soybean 7S	β-glucuronidase	1
R35H7	*fad2-3*	35S	*CFAH12*	2
TA1...TA14	WT	35S	*CFAH12*	3
TB1...TB10	WT	35S	*LFAH12*	3
TC1...TC12	*B. napus*	Napin	*CFAH12*	3
TD1...TD29	*B. napus*	Napin	*LFAH12*	3
TE1...TE12	*fad2-3*	35S	*LFAH12*	3
TF1...TF21	WT	*LFAH12*	*LFAH12*	3
TG1...TG32	WT	*LFAH12*	β-glucuronidase	3

WT is the columbia wild type of *Arabidopsis*.
Sources: 1, Hirai *et al.* (1994); 2, Broun and Somerville (1997); 3, this study.

A genomic clone of the putative oleate hydroxylase was isolated by using *DLS1* to probe filter lifts of a genomic library of *L. fendleri*. The putative coding region was identified by comparison of the nucleotide sequence flanking the region of DNA corresponding to *DLS1* with the sequence of the *FAD2* gene from *Arabidopsis*. The nucleotide sequence of a region of 3670 bp containing the gene was deposited in GenBank as accession number AF016103. This included 2217 bp of 5′ flanking sequence, an 1152 bp open reading frame lacking introns, and a 302 bp 3′ flanking region. The gene, designated *LFAH12*, encodes a polypeptide of 384 amino acids which shows 81% sequence identity to the *Arabidopsis* FAD2 desaturase and 71% sequence identity to the castor oleate hydroxylase. The fact that LFAH12 is more similar to FAD2 than to CFAH12 suggests that the *L. fendleri* and castor genes have evolved independently from desaturases, rather than from an ancestral hydroxylase.

Evidence that *LFAH12* encoded an oleate 12-hydroxylase was obtained by placing the gene under the control of the CaMV 35S promoter in transgenic *Arabidopsis* plants. Ten independent transgenic lines designated TB1 to TB10 were obtained (Table 1). Ricinoleic acid represented about 4% of total seed fatty acids in line TB10, the transgenic line with the highest amount of hydroxy fatty acids (Table 2). Thus, we concluded that *LFAH12* encodes an oleate 12-hydroxylase. Transgenic seeds also accumulated densipolic, lesquerolic and traces of auricolic acids. Hydroxy fatty acids did not accumulate in vegetative organs of transgenic plants expressing *LFAH12* (Table 2). A similar result was previously obtained with transgenic plants expressing the castor hydroxylase (Broun and Somerville, 1997).

The *fad2* mutant of *Arabidopsis* accumulates elevated amounts of 18:1 because of a defect in microsomal oleate 12-desaturase. In order to test the effect of substrate availability on the accumulation of hydroxy fatty acids, 12 transgenic lines of the *fad2-3* mutant containing the *LFAH12*

Table 2. Fatty acid composition of transgenic plants expressing *LFAH12* and *CFAH12*

Seeds					Leaves			Roots		
Fatty acid	WT	TB10	TA14	TF21	WT	TB10	TA14	WT	TB10	TA14
–					mol%					
16:0	9	8	9.6	8.7	12.6	12.4	12.9	21.4	20.7	20.4
16:3	0	0	0	0	13.2	15	13.9	0	0	0
18:0	3.5	3.7	3.6	3.5	1	0.9	1.1	1.1	1.6	1.5
18:1	17.1	33.5	32.8	26	3.1	4.1	3.8	2.8	4.3	3.4
18:2	28.8	15.4	15.2	18.7	14.3	13	14.4	29.6	28.1	31.9
18:3	15.9	9.6	9.9	11	47.4	46 4	6.5	27	24.5	22
20:0	2.1	1.9	1.3	1.8	0	0	0	0	0	0
20:1	18	16.4	16.2	15.9	0	0	0	0	0	0
18:1-OH	0	3.9	3.4	5.9	0	0	0	0	0	0
18:2-OH	0	5.2	4.9	5.6	0	0	0	0	0	0
20:1-OH	0	0.9	1.3	1.3	0	0	0	0	0	0

Abbreviations: 16:0 palmitic acid; 16:3, *cis*-hexadecetrienoic (ω3,6,9); 18:0, stearic acid; 18:1, *cis*-oleic (ω9); 18:1-OH, ricinoleic acid; 18:2, *cis*-linoleic (ω6,9); 18:2-OH, densipolic acid; 18:3, *cis*-linolenic (ω3,6,9); 20:1, eicosenoic acid (ω9); 20:1-OH, lesquerolic acid.

Table 3. Fatty acid composition of transgenic plants of the *Arabidopsis fad2–3* mutant expressing *LFAH12* and *CFAH12*

Seeds			Leaves		Roots		
	fad2–3	TE12	*fad2–3*	TE12	*fad2–3*	TE12	R35H7
			mol%				
16:0	4.1	4.11	11.4	10.9	9.5	14.8	12.5
16:3	0	0	16.4	17	0	0	0
18:0	2.7	3.5	0.7	0.5	1.2	1.5	1.6
18:1	40.6	44.8	19.7	20.3	55.4	27.9	47.5
18:2	7.1	3.7	4	3.7	3.9	16.7	4.4
18:3	11.4	6.7	38.2	39.8	11	16.4	13.2
20:0	1.4	1.5	0	0	0	0	0
20:1	20	15.2	0	0	0	0	0
18:1-OH	0	6.5	0	0	0	0	0
18:2-OH	0	8.5	0	0	0	0	0
20:1-OH	0	1.5	0	0	0	0	0

gene under control of the CaMV 35S promoter were produced (designated TE1 to TE12 in Table 1). Three TE lines had significantly higher levels of hydroxy fatty acids than were obtained by using the same construct to produce transgenic plants in the wild-type background. The fatty acid composition of one of these lines, TE12, is shown in Table 3. Thus, it appears that one of the factors limiting accumulation of hydroxy fatty acids in seeds of transgenic *Arabidopsis* plants is the competition between the oleate desaturase and the oleate hydroxylase for 18:1.

Activities of the L. fendleri *and castor hydroxylases are comparable in transgenic* Arabidopsis *seeds*

In an effort to compare the *in vivo* activities of the *L. fendleri* and the castor hydroxylases, we measured the amounts of ricinoleic, densipolic and lesquerolic acids in seeds from a series of independent transgenic *Arabidopsis* plants expressing either the *L. fendleri* or castor genes under the control of the CaMV 35S promoter (Table 1). As shown in Figure 2, the two populations of transgenics have very similar amounts of hydroxylated fatty acids, ranging from about 0.1% to about 9% of total fatty acids. This suggests that the *in vivo* activity of the two enzymes is similar in *Arabidopsis*. The ratios of ricinoleic to densipolic acid was roughly constant among transgenic lines (about 1:1). However, the ratio of ricinoleic to lesquerolic acids was slightly higher in lines expressing the *L. fendleri* gene than in lines expressing the castor gene (2.3 ± 0.5% and 3.7 ± 0.8%, respectively).

It has previously been observed that expression of the castor hydroxylase in transgenic *Arabidopsis* plants causes an apparent inhibition of the FAD2 oleate desaturase (Broun and Somerville, 1997). The mechanism responsible for this effect is not known. A similar effect was observed in seed lipids, but not leaf or root lipids, of plants expressing the *L. fendleri* gene (Table 2). This was apparent as an increase in the amount of 18:1 from ~ 17% in the wild-type control

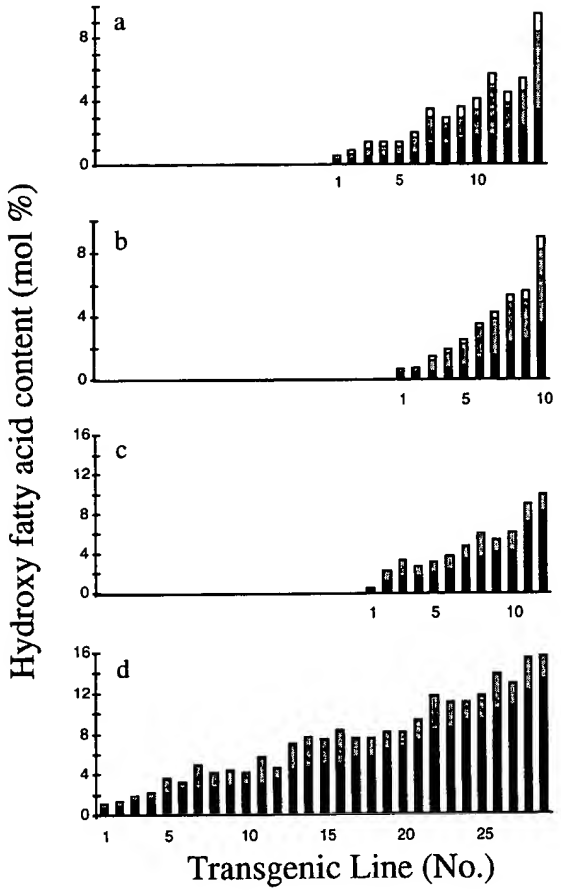

Figure 2. Hydroxy fatty acid content of transgenic seeds, expressed as a percentage of total seed fatty acids.
(a) *Arabidopsis* line TA14 (35S-CFAH12) or (b) line TB10 (35SLFAH12), (c) *B. napus* line TC12 (pNap-CFAH12) or (d) line TD29 (pNap-LFAH12). The values for *Arabidopsis* were averages for samples of 25 R1 seeds. The values for *B. napus* were averages for 20 R1 seeds. The amount of ricinoleic is indicated by dark grey bars, densipolic is indicated by light grey bars and lesquerolic is indicated by white bars.

to about 33% in the plants containing LFAH12. The increase in 18:1 was accompanied by a decrease in the amount of 18:2, suggesting that the effect was due to inhibition of FAD2 activity.

The expression of LFAH12 and CFAH12 in transgenic B. napus *plants results in hydroxy fatty acid accumulation*

The coding regions of the castor and *L. fendleri* genes were placed under the control of a strong seed-specific promoter from the *Brassica rapa* napin gene (Kridl *et al.*, 1991). The resulting cassettes pNap-LFAH12 and pNap-CFAH12 were introduced into *B. napus* plants by *Agrobacterium*-mediated transformation. Twelve transformants were obtained containing the castor gene, and 29 containing the *L. fendleri* gene (Table 1). The presence of either gene resulted in the accumulation of hydroxy fatty acids in seeds (Table 4). The proportions of ricinoleic and densipolic acid contents in the individual transgenic lines expressing the *L. fendleri* or the castor gene are shown in Figure 2(c and d). Lesquerolic acid was not detected in these plants because of the absence of oleate elongase activity. As in the case of *Arabidopsis* transgenic lines, the data suggest that *in vivo* activities of the *L. fendleri* and castor hydroxylases are similar.

Table 4. Fatty acid composition of transgenic *B. napus* plants expressing *LFAH12* (TC29) and *CFAH12* (TD29)

Fatty acid	WT	TC12	TD29
		mol%	
16:0	6.9	5.1	5.5
18:0	1.4	1.7	1.8
18:1	51.6	59.1	64
18:2	26.4	11.3	9.9
18:3	9.4	3.4	3.8
18:1-OH	0	13.3	8.2
18:2-OH	0	2.3	1.7

The relative proportions of ricinoleic and densipolic acids were different in transgenic *B. napus* and in transgenic *Arabidopsis*. In *B. napus*, the average ricinoleic:densipolic ratio was about 2.5 (2.9 in the case of the pNap-LFAH12 plants, 2.3 in the case of pNap-CFAH12), which was significantly higher than from the corresponding transgenic *Arabidopsis* plants. This observation suggests that the *Arabidopsis* Δ15-linoleate desaturase is more efficient at desaturating ricinoleic acid than the *B. napus* homolog.

Apparent discrepancy between hydroxylase protein and mRNA levels in transgenic plants

Hydroxy fatty acids did not accumulate in roots of transgenic plants expressing 35S-CFAH12 or 35S-LFAH12. However, levels of *CFAH12* or *LFAH12* messenger RNA were relatively high in roots of 35S-CFAH12 and 35S-LFAH12 transgenic plants (Figure 3). Phosphorimager quantitation of the hybridization signals on the Northern blot in Figure 3(b) indicated that the amount of *CFAH12* or *LFAH12* mRNA accumulation in transgenic roots was ~ 50% as high as the level of *CFAH12* mRNA accumulation in developing castor bean endosperm. The correlation between mRNA levels and protein accumulation was examined by using an antibody raised against the CFAH12 hydroxylase to probe Western blots. An abundant polypeptide of the expected molecular weight (44 kDa) was detected in extracts of developing castor seeds (Figure 3a). However, no signal was detectable in root extracts from transgenic line TB10. Although we cannot rule out the possibility that

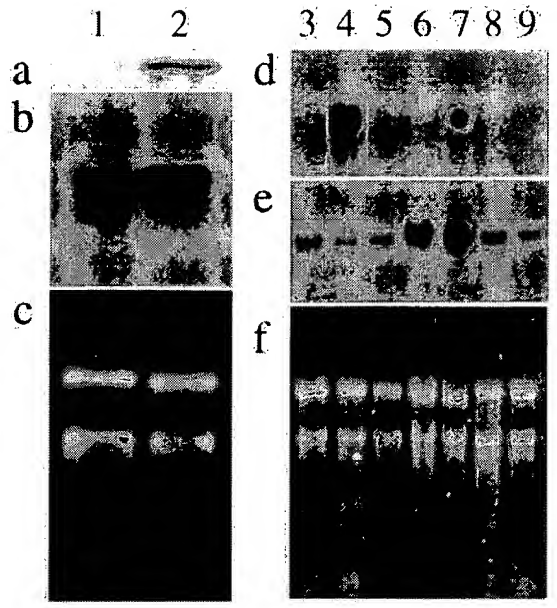

Figure 3. Comparison of mRNA and protein levels in transgenic plants.
(a) Western blot of microsomal proteins from roots of TA14 (35S-CFAH12) transgenic *Arabidopsis* plants (lane 1) and castor developing endosperm (lane 2).
(b) Northern blot of RNA from roots of transgenic lineTA14 (35S-CFAH12) (lane 1) and castor developing endosperm (lane 2), probed with part of a CFAH12 cDNA.
(c) Ethidium bromide stained gel used to produce blot shown in (b).
(d) Northern blot of total RNA from roots of WT (lane 3), roots of TB10 (35S-LFAH12) (lane 4), roots of TF21 (pLesq-LFAH12) (lane 5), siliques of WT (lane 6), siliques of TF21 (pLesq-LFAH12) (lane 7), flowers of WT (lane 8), flowers of TF21 (pLesq-LFAH12) (lane 9), probed with a portion of the LFAH12 gene.
(e) The same Northern blot shown in d reprobed with the FAD2 cDNA.
(f) Ethidium bromide stained gel used to produce blot shown in d and e.

protein instability in root but not in castor bean extracts could account in part for the observed difference, this result raises the intriguing possibility that a post-transcriptional mechanism prevents the enzyme from accumulating in transgenic roots.

These results suggest that accumulation of the hydroxylase protein, in addition to fatty acid breakdown, may be a limiting factor to hydroxy fatty acid accumulation in vegetative organs.

Measurement of hydroxylase activity

In order to establish if a functional hydroxylase enzyme accumulated in transgenic plants expressing *LFAH12* and *CFAH12*, hydroxylase activity was directly measured in root extracts. One 35S-LFAH12 plant (TA14) and one 35S-CFAH12 plant (TB10) were chosen for analysis based on the similarity of hydroxy fatty acids levels in their seeds (Table 2). The levels of oleate desaturase and oleate hydroxylase activity of extracts from roots of lines TA14 and TB10 were similar in both plants (Table 5). In both cases, desaturase activity was lower in extracts of the transgenics than in extracts from wild-type plants. This is generally consistent with the observation that seed oleate desaturase activity appears to be lower in the transgenics than in wild type (Table 2).

Table 5. Hydroxylase and desaturase activity of extracts from roots of transgenic *Arabidopsis* plants. Because the substrate provided in the assay (acyl-CoA) is not thought to be the direct substrate for the desaturase or hydroxylase, the amount of product reflects both acyltransferase activity and desaturase or hydroxylase activity

Genotype	Desaturase activity	Hydroxylase activity
	pmol product/mg protein/h	
WT	5.72 ± 0.62	nd
TB10	0.90 ± 0.36	0.96 ± 0.25
TA14	1.40 ± 0.05	0.68 ± 0.07
fad2	0.64 ± 0.06	nd
TE12	0.36 ± 0.24	0.28 ± 0.06

We were not able to detect desaturase or hydroxylase activity in leaf extracts. However, ^{14}C acetate feeding of detached leaves from TA14 plants resulted in the accumulation of low levels of labelled ricinoleic acid in the free fatty acid fraction (not shown). These *in vitro* results indicate that the roots, and probably the leaves, of transgenic plants contain significant levels of hydroxylase activity. However, the fact that vegetative organs of transgenic plants do not contain detectable levels of hydroxy fatty acids suggests that hydroxy fatty acids are synthesized and broken down at a higher rate than 18:1 or 18:2 or 18:3.

The L. fendleri *hydroxylase has desaturase activity*

In order to examine the possibility that LFAH12 exhibited desaturase activity, the *LFAH12* gene, under the control of the CaMV 35S promoter, was introduced into the *fad2-3* mutant of *Arabidopsis* (Table 1). The fatty acid composition of the transgenic line accumulating the highest levels of hydroxy fatty acids, line TE12, is shown in Table 3. The leaf fatty acid composition of transgenic plants does not differ from control *fad2-3* plants, although in the same plants expression of the *LFAH12* gene results in the accumulation of hydroxy fatty acids, up to 16% of seed fatty acids. In contrast, the root fatty acid composition of the transgenic line was dramatically different from that of the untransformed mutant. Levels of 18:1 were lower and 18:2 levels higher, giving transgenic roots an intermediate phenotype between mutant and WT plants (Table 3). Unlike roots, seeds did not show signs of suppression. This may be due to the fact that the activity of the CaMV 35S promoter is significantly lower in developing seeds than in roots.

We conclude from this experiment that the *L. fendleri*

hydroxylase has significant desaturase activity. In contrast, the root fatty acid composition of a transgenic 35S-CFAH12 *fad2-3* line, R35H7, which was previously shown to accumulate up to 9% hydroxy fatty acids in its seeds (Broun and Somerville, 1997), was indistinguishable from untransformed *fad2-3* roots (Table 3). Thus, we could find no evidence that the castor hydroxylase has desaturase activity.

The LFAH12 *promoter is early and highly active in developing seeds of transgenic* Arabidopsis *plants*

Relatively few early seed-specific promoters are currently available for genetic engineering of seed lipid composition. Therefore, we examined the possible utility of the *L. fendleri* hydroxylase promoter in this respect. A genomic fragment containing the *LFAH12* coding region and an additional 2.2 kb of upstream sequence (pLesq-LFAH12) was used to produce 21 transgenic lines of wild-type *Arabidopsis* (Table 1). The ricinoleic and densipolic acid contents of seeds from the line, TF21, accumulating the highest levels of hydroxy fatty acids are shown in Table 2. Accumulation of hydroxy fatty acids in plants expressing the *L. fendleri* gene under control of its own promoter was, on average, 3.1 times higher than in plants transformed with the 35S-LFAH12 construct (results not presented). The levels of hydroxy fatty acids were also similar to those in previously described plants transformed with pNapin-CFAH12 (Broun and Somerville, 1997). Since the two hydroxylases have comparable activities in seeds of transgenic *Arabidopsis* plants, we conclude that the *L. fendleri* promoter is highly active in developing seeds of *Arabidopsis* plants.

In order to characterize the developmental pattern of promoter activity, the same 2.2 kb promoter fragment was placed upstream of the β-glucuronidase (GUS) gene and introduced into *Arabidopsis* plants (Table 1). Histochemical staining was performed for three independent transformants (TG30, TG31 and TG32) on leaves, stems, inflorescences and embryos at different stages of development (Figure 4). There was no staining in leaves, stems and flowers, light staining of young siliques, and strong staining of embryos. We also performed Northern analysis of roots, flowers and siliques of the pLesq-LFAH12 line (TF21) that had the highest level of expression of *FAH12*, using *LFAH12* as a probe (Figure 3d). Under our conditions, there was weak cross-hybridization between *LFAH12* and *Arabidopsis FAD2* message. However, there was no difference between WT and transgenic plants in roots and flowers, whereas the *L. fendleri* probe detected a strong signal in maturing siliques of transgenic plants (Figure 3d). Based on these results, it appears that the *L. fendleri* promoter region can be used to cause seed-specific expression of foreign genes.

Because efforts to genetically engineer seed lipid composition benefit from the availability of a strong early seed-specific promoter, we compared the timing and strength of expression of the *LFAH12* promoter with a seed storage protein promoter. Hirai *et al.* (1994) have previously characterized transgenic *Arabidopsis* plants that expressed the GUS gene under control of the promoter from the alpha subunit of the soybean β-conglycinin (7S). Histochemical staining was performed on embryos of transgenic line TG32 and line SNTα133, which contained β-glucuronidase under control of the 7S promoter (Table 1). Both transgenic lines were chosen for their high level of GUS expression. Staining patterns of representative *Arabidopsis* embryos from lines SNTα133 and TG32 are shown in Figure 4. These results indicate that the *L. fendleri* promoter appears to be active significantly earlier than the 7S promoter, and that the onset of activity coincides with that of storage lipid accumulation in *Arabidopsis*.

Discussion

We report here the isolation and characterization of *LFAH12*, an oleate 12-hydroxylase gene, from *L. fendleri*. The similarity of LFAH12 to microsomal oleate-12 desaturases is striking; the deduced amino acid sequence of the *L. fendleri* hydroxylase gene is 81% identical to the FAD2 desaturase from *Arabidopsis*, and between 70 and 80% identical to a number of other desaturase genes. We have also isolated a partial cDNA sequence from *L. fendleri* (DLS2) which encodes a protein that is ~ 90% identical to LFAH12. The corresponding transcript is more abundant in leaves than in seeds, which suggests it encodes an oleate desaturase (Figure 1). These observations support the hypothesis that *LFAH12* has evolved from a desaturase gene. This hypothesis is also supported by the observation that LFAH12 exhibits both desaturase and hydroxylase activity in transgenic *Arabidopsis*. In this respect, LFAH12 differs from CFAH12, the castor enzyme, which does not exhibit detectable desaturase activity.

We have established that transgenic *B. napus* and *Arabidopsis* plants expressing LFAH12 or CFAH12 accumulate hydroxy fatty acids to the same extent. This suggests that LFAH12 and CFAH12 have similar activities *in vivo*. However, castor and *L. fendleri* seed fatty acid compositions are substantially different. Castor seeds contain between 80% and 90% ricinoleic acid, whereas *L. fendleri* accumulates about 0.5% ricinoleic, 60% lesquerolic and 5% auricolic acids (Hayes *et al.*, 1995). Castor seeds do not accumulate long chain fatty acids, as opposed to *L. fendleri* seeds, which contain about 0.5% of 20:1 (Atsmon, 1989; Hayes *et al.*, 1995). Because the ratios of ricinoleic to lesquerolic acids are similar in transgenic *Arabidopsis* plants expressing either *CFAH12* or *LFAH12*, it does not appear that substrate specificities of the *L. fendleri* and the castor enzymes are substantially different. Thus, it seems likely that the differences in seed fatty acid accumulation

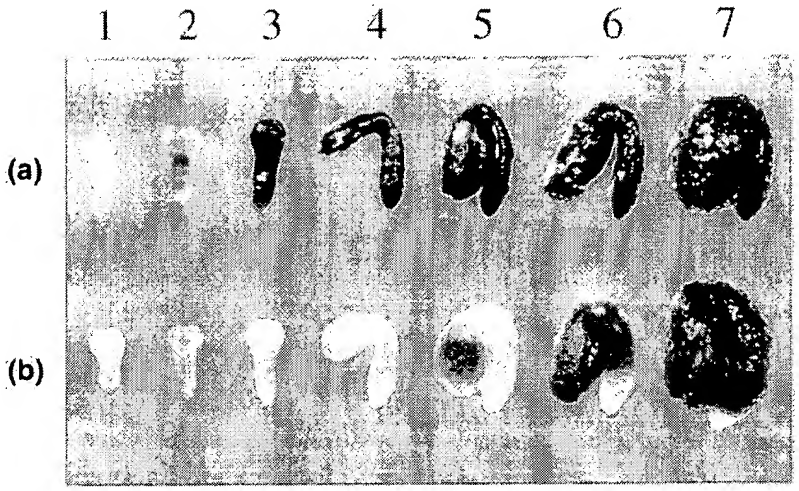

Figure 4. Histochemical staining of embryos at various developmental stages from transgenic *Arabidopsis* plants expressing the GUS gene fused to the LFAH12 promoter. (a) Embryos from line TG32 (pLesq-GUS); (b) embryos from line SNTα133. The stages numbered 1–7 were, respectively, 4 days after pollination (DAP); 4–5 DAP; 5 DAP; 5–6 DAP; 6–7 DAP; 7–8 DAP; and 10–12 DAP.

between castor and *L. fendleri* is primarily due to the presence of fatty acid elongase activity in *L. fendleri*.

On the basis of labelling studies it has been proposed that the principal route of lesqueroleate synthesis is via elongation of ricinoleate rather than by hydroxylation of 20:1 (Reed *et al.*, 1997). Our results indicated that the *L. fendleri* hydroxylase does not have greater preference for 20:1 than for 18:1. If the principal route of synthesis of lesquerolic acid were via hydroxylation of 20:1, preference for 20:1 by the *L. fendleri* hydroxylase might be expected since lesquerolic acid is the major fatty acid in the seeds of *L. fendleri*. In this respect, our results are consistent with the proposal by Reed *et al.*(1997).

We were able to detect desaturase activity of LFAH12 by expressing it in transgenic *fad2* plants. However, in *L. fendleri* seeds, where expression of *LFAH12* is strong and where we have evidence that a desaturase gene is also expressed, 18:2 accumulates to fairly low levels (about 6%). This suggests that transfer of hydroxy fatty acids to the triacylglycerol pool is greatly favoured. Preference of acyltransferases for ricinoleic acid has been demonstrated *in vitro* in assays of extracts from castor developing endosperm (Bafor *et al.*, 1991; Wiberg *et al.*, 1994). Our data are consistent with the idea that a similar mechanism operates with the different hydroxy fatty acids in *L. fendleri* seeds.

Another puzzling observation related to LFAH12 desaturase activity is that expression of LFAH12 in transgenic *fad2* plants resulted in suppression of the mutant phenotype in roots but not in leaves. The opposite might be expected, since *fad2* leaves have chloroplastic oleate desaturase activity, and the reduction in 18:2 and 18:3 accumulation compared to WT is not as dramatic as in roots of the mutant. It is possible that although some level of hydroxylase activity is detectable in this organ, enzyme accumulation or activity is more severely limited in leaves that in roots of transgenic plants. Western analysis of leaf extracts with high affinity antibodies to the *L. fendleri* hydroxylase should help resolve this question.

Measurements of hydroxylase activity in crude extracts of roots from transgenic plants expressing either the castor or the *L. fendleri* gene indicate the presence of hydroxylase activity. Indeed, when root microsomal membranes were incubated in the presence of ^{14}C oleoyl-CoA, linoleic acid and ricinoleic acid were labelled to the same extent in plants expressing the *L. fendleri* hydroxylase. Because linoleic acid and linolenic acids accumulate to over 50% of root fatty acids, whereas ricinoleic is not detectable in transgenic roots, an efficient mechanism is clearly limiting hydroxy fatty acid accumulation in transgenic plants. One possible mechanism is that hydroxy fatty acids are detected as being abnormal and are selectively removed and metabolized. Phospholipase activity has been characterized in the seeds of certain plant species, which preferentially targets membrane lipids containing unusual fatty acids (Banas *et al.*, 1992; Stahl *et al.*, 1995).

Western analysis of root microsomal proteins shows that high levels of *CFAH12* mRNA in castor and in transgenic plants does not translate into proportional levels of protein accumulation. Thus, accumulation of the castor hydroxylase appears to be limited post-transcriptionally in roots of transgenic *Arabidopsis* plants. This situation is reminiscent of recent studies concerning the overexpression of HMG CoA reductase in *Arabidopsis* plants. Although HMG CoA reductase mRNA levels were dramatically increased upon introduction of the transgene, no effect was observed on protein accumulation (Re *et al.*, 1995). In the case of the hydroxylase, which is foreign to *Arabidopsis*, enzyme accumulation may be controlled by a mechanism which normally acts on the FAD2 enzyme, because of the high level of identity between the two

proteins. This raises the intriguing possibility that desaturation levels may be controlled in part through post-transcriptional regulation of desaturase enzymes such as FAD2.

In addition to encoding a unique enzyme, the *L. fendleri* gene appears to be controlled by an interesting promoter of a novel class. This promoter is active only in developing seeds in both *L. fendleri* and *Arabidopsis*, at the time of storage lipid accumulation. At present, much of the information about seed-specific gene expression has been derived from studies of genes encoding storage proteins (reviewed by Bevan *et al.*, 1993). For instance, DNA sequences that confer embryo-specific expression of the soybean conglycinin promoter in transgenic plants have been identified (Chen *et al.* 1988). Similarly, the storage protein napin is one of the major protein components of *Brassica napus* seeds. A 152 bp fragment from the napin promoter directed strong expression of the β-glucuronidase reporter gene in mature tobacco seeds (Stalberg *et al.*, 1996). The napin promoter has been used to control expression of genes in transgenic plants designed to produce novel fatty acids (Voelker *et al.*, 1996). However, because storage lipid accumulation begins substantially before the maximal level of expression of the napin or other storage protein genes are reached (Post-Beittenmiller *et al.*, 1992), the promoters of storage protein genes are not ideal for controlling expression of genes related to storage lipid accumulation. *LFAH12* is one of the first genes involved in lipid metabolism which is expressed at high levels in a seed-specific manner. For this reason, its promoter should be useful to control the expression of genes for enzymes involved in modification of the seed lipid composition of crop plants.

Experimental procedures

Plant material

L. fendleri seeds were obtained from K. Carlson, Oregon State University, Corvallis, OR, USA. The Westar cultivar of *B. napus* was used. All *Arabidopsis thaliana* lines used were from the ecotype Columbia. The *Arabidopsis fad2–3* mutant line has been described previously (Miquel and Browse, 1992). All transgenic lines are described in Table 1. The transgenic lines SNTα133, which contains the β-glucuronidase gene under control of the soybean 7S promoter, was obtained from Satoshi Naito, Hokkaido University, Hokkaido, Japan.

DNA clones

The *FAD2* clone pYesFAD2 was obtained from John Shanklin (Brookhaven National Laboratory, Uptown, NY, USA). It consisted of a PCR-amplified fragment comprising the complete coding region, cloned into the pYES2 vector (InVitrogen). The clone pA4 carrying the castor hydroxylase gene *CFAH12* has been described previously (van de Loo *et al.*, 1995).

RNA manipulations

Arabidopsis RNA from various tissues was obtained using the method from Verwoerd *et al.* (1989). RNA from *L. fendleri* leaves and seeds was extracted using an RNeasy kit from Qiagen. For Northern analysis, 5 μg of total RNA was run in each lane of an agarose gel containing formaldehyde. RNA was subsequently transferred to an Optitran supported nitrocellulose membrane (Schleicher and Schuell) according to the manufacturer's protocol. Northern hybridization was performed in 5× SSC, 10× Denhardt's solution, 0.1 M KPO_4 pH 6.8, 10% Dextran Sulfate, 30% formamide and 100 μg ml^{-1} salmon sperm DNA, using PCR-amplified inserts from pYesFAD2, pA4, *DLS1* and *DLS2* as probes. Blots were washed in 0.2 × SSC, 0.5% SDS for 30 min at 52°C, then for 15 min in 2 × SSC at 65°C.

For reverse transcriptase PCR 5 μg of *L. fendleri* seed RNA were denatured for 10 min at 65°C, then annealed with 1 μg oligo(dT18) for 10 min at 25°C in the presence of RNAse inhibitors. The RNA was then incubated for 1 h at 37°C in the presence of 50 mM Tris–HCl pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM MgCl2, 500 μM of each dNTP and 50 U RNase H- MMLV reverse transcriptase (Stratagene), in a final volume of 40 μl. 5 μl of the previous reaction were added to a PCR reaction containing 200 μM dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 9), 0.1% (v/v) Triton X-100, 1.5 mM $MgCl_2$, 3% (v/v) formamide, 125 pmol of each primer, 2.5 U of Taq polymerase (Promega), to a final volume of 50 μl.

Amplifications conditions were: 4 min denaturation step at 94°C, followed by 30 cycles of 92°C for 1 min, 50°C for 1 min, 72°C for 2 min, concluding with a final extension step at 72°C for 5 min. Oligonucleotide primers were designed by choosing regions of high deduced amino acid sequence homology between the castor hydroxylase and the *Arabidopsis* desaturase. The sequence of the mixed oligonucleotides was: TAYWSNCAYMGNMGNCAYCA (Deg1) and RTGRTGNGCNACRTGNGTRTC (Deg2), where Y = C + T, W = A + T, S = *G* + C, N = A + *G* + C + T, M = A + C, R = A + G. The reaction products were run on an agarose gel, and a fragment of the expected size (~ 540 bp) was purified. About 10 ng of the resulting DNA was used in a second round of PCR amplification using the same conditions, but for 15 cycles only. The amplified products were gel-purified, and cloned into pGEM-T (Promega), according to the manufacturer's conditions.

DNA manipulations

For the construction of an *L. fendleri* genomic library, genomic DNA was prepared from young leaves of *L. fendleri* as described by Murray and Thompson (1980). A *Sau*3AI-partial digest genomic library constructed in the vector λDashII (Stratagene) was prepared by partially digesting 500 μg of DNA, size-selecting the DNA on a sucrose gradient (Sambrook *et al.*, 1989), and ligating the DNA (12 kb average size) to the *Bam*HI-digested arms of λDashII. The entire ligation was packaged according to the manufacturer's conditions and plated on *E. coli* strain XL1-Blue MRA-P2 (Stratagene). This yielded 5 × 10^5 primary recombinant clones. After amplification according to the manufacturer's recommendations, the genomic library was plated on *E. coli* XL1-Blue and about 150 000 clones were screened using the amplified insert from DLS1 as a probe. Seven clones were obtained and suitable portions of the corresponding inserts subcloned (see below).

DNA constructs

p35SLFAH12 was constructed as follows. One of the seven phage clones purified from the *L. fendleri* genomic library (see above)

was cut with *Eco*RI, and a 1.4 kb fragment was subcloned into the pBluescript KS (Stratagene), resulting in the vector pLFAH12-1. pLFAH12-1 was cut with *Sal*I, treated with Klenow to blunt receding ends, then cut with *Sac*I. The insert fragment was ligated to a gel-purified vector fragment from pBI121 cut with *Sma*I and *Sac*I. This vector was used to introduce *LFAH12* under the control of the CaMV 35S promoter (35S-LFAH12 cassette) into *Arabidopsis* plants.

pLesq-LFAH12. A second phage clone was cut with *Sal*I, and the entire insert ligated to the primary vector pSLJ44024 (Jones *et al.*, 1992) cut with *Xho*I, resulting in the vector SLJpLesq-LFAH12. This vector was used to introduce the *L. fendleri* gene under the control of its own promoter (pLesq-LFAH12 cassette) into *Arabidopsis* plants.

pNap-LFAH12. A pBluescript derivative, pBSNapin, which contains the PCR amplified Napin promoter (Broun and Somerville, 1997), was cut with *Stu*I and *Sac*I. pLFAH12-1 was cut with *Sal*I, treated with Klenow to blunt receding ends, then cut with *Sac*I. The insert fragment was ligated to the pBSNapin vector fragment. The resulting vector was cut with *Not*I, and an insert fragment comprising the Napin promoter and the *LFAH12* coding region was cloned into the vector pMON17227, a derivative of pBIN19 carrying an *Agrobacterium* EPSP synthase gene, which confers resistance to glyphosate (Padgette *et al.*, 1995). pNap-LFAH12 was used to introduce *LFAH12* under the control of the Napin promoter (pNap-LFAH12cassette) into *B. napus* plants.

pNapCFAH12. In a first step, pFL2 (van de Loo *et al.* 1995) was cut with *Not*I, submitted to Klenow treatment, then religated. This eliminated the *Not*I site in the vector. In a second step, pFL2 was cut with *Bam*HI and *Xba*I, and the insert fragment containing the coding region of the castor hydroxylase gene was cloned into pBSNap cut with *Bgl*II and *Xba*I. The resulting vector was cut with *Not*I and the insert cloned into pMON71227, giving pNap-CFAH12. This vector was used to introduce *CFAH12* under the control of the Napin promoter (pNap-CFAH12 cassette) into *B. napus* plants.

pLesq-GUS, a binary vector carrying the LFAH12 *promoter fused to the* GUS *gene.* In a first step, pLesq-LFAH12 was cut with *Hin*dIII and *Eco*RI, and a 2.2 kb insert fragment ligated to pBluescript. In a second step, the resulting vector was cut with *Hin*dIII and *Bam*HI, and ligated to pBI101 to obtain pLesq-GUS. This vector was used to transform *Arabidopsis* plants with the pLesq-GUS cassette.

Antibodies against the castor hydroxylase

A portion of *CFAH12* encoding a 100 amino acid polypeptide at the C-arboxy terminus of the enzyme was subcloned into the expression vector pQE32 (Qiagen). To this effect, pFL2 was cut with *Bam*HI and *Sac*I, and the insert fragment was gel-purified. This fragment was then cut again with *Sau*3AI, and the resulting digest was added to a ligation reaction containing pQE32 cut with *Bam*HI and *Sac*I. The resulting vector, pQECFAH12, was introduced into the *E. coli* strain M15. Cultures were induced and the expressed polypeptide was purified from the insoluble fraction by passage through a Nickel column under denaturing conditions according to the manufacturer's protocol. The protein was then purified by electro-elution from an SDS–PAGE gel. Rabbits were immunized by serial injections of the solubilized protein.

For Western analysis, 20 μg of microsomal proteins (see preparation below) was loaded on SDS–PAGE gels, then transferred using a semi-dry blotter to PVDF membranes (Millipore). Western analysis was then performed as in Jabben *et al.* (1989). A 1/1000 dilution of crude immune and pre-immune antisera was used for primary antibody incubations. Proteins were detected in a calorimetric assay using goat anti-rabbit IgG conjugated to alkaline phosphatase.

Enzyme assays

Root microsomes were prepared as in Miquel and Browse (1992), with some modifications. The extraction buffer contained 2.5 mM NADH and catalase was 10 000 U ml^{-1} instead of 2000 U ml^{-1}. After centrifugation at 100 000 *g*, microsomal membranes were rinsed in desaturase reaction buffer (also containing 10 000 U ml^{-1} catalase) before being dispersed in the same buffer to a final concentration of ~ 0.5 mg ml^{-1} microsomal protein. The membranes were then incubated for 1 h in the presence of 85 000 d.p.m. ^{14}C-oleoyl CoA (52 Ci mol^{-1}), and the labelled lipids were extracted after addition to the reaction of an equal volume of 2 M NaCl, 0.2 N HCl and 2 ml of chloroform/methanol (1:1). The chloroform phase was recovered, dried under nitrogen, and the fatty acids were transmethylated in 1 N methanolic HCl for 1 h at 80°C. After addition of an equal volume of 0.9% NaCl, the fatty acid methyl esters (FAME) were extracted into hexane. The hexane was subsequently evaporated under nitrogen, and the FAME were redissolved in 50 μl chloroform. The FAME were then separated along side standards by argentation TLC as in Miquel and Browse (1992), using hexane/ethyl ether (80:20) as the mobile phase. After drying, the plates were exposed to phosphorimager cassettes.

Protein quantitation

Proteins were quantitated using Bradford assay reagents (Biorad) or a TCA kit (Pierce) and BSA as a standard.

Gas chromatography

Fatty acids from different plant tissues were extracted and derivatized as in Broun and Somerville (1997). Fatty acid methyl esters from 0.1 to 0.5 g fresh leaf or root tissue or 0.7 mg seed material were dissolved in 1 ml hexane and 1 μl of the extracts was analysed by gas chromatography as described by Broun and Somerville (1997).

Histochemical staining of GUS activity

Tissues were incubated in staining buffer (50 mM KPO_4 buffer pH 7.0 containing 20% methanol, 0.5% Triton X-100, 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide and 3 mM X-Gluc). Samples were placed in a vacuum chamber at 650 mmHg for 2 min then incubated for 15 h at 37°C. Following staining, samples were cleared by successive 5 min immersions in 20, 40, 60 and 70% ethanol

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DLS1 and *DLS2* have GenBank accession numbers AF016104 and AF016105, respectively. The *L. fendleri* hydroxylase sequence has GenBank accession number AF016103.